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Endothelin-1 inhibits cytokine-stimulated transcription of inducible nitric oxide synthase in glomerular mesangial cells

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Endothelin-1 inhibits cytokine-stimulated transcription of inducible nitric oxide synthase in glomerular mesangial cells. Endothelin-1 (ET-1) is a potent vasoconstrictor while nitric oxide (NO) has strong vasodilatory effects. Recent studies have indicated that vasoconstrictors and NO may mutually modulate their production and/or activity, thus regulating each other in the context of microcirculatory maintenance. We examined the question whether ET-1 may affect NO formation by controlling the expression of the inducible isoform of the NO synthase (iNOS) in cultured rat glomerular mesangial cells (MCs), as induced by the inflammatory cytokines, tumor necrosis factor-α (TNF-α) plus interleukin-1β (IL-1β). We found that ET-1 in MCs markedly reduced cytokine-induced NO production (measured as stable NO_2^-) and inhibited the expression of iNOS mRNA (Northern blot analysis) and of iNOS protein (Western blotting). Inhibition of cytokine-stimulated iNOS mRNA expression by ET-1 was almost complete at the level of gene transcription while post-transcriptional effects were not detected. The ET_A receptor antagonist BQ-123 blocked the inhibitory effect of ET-1. The ET_A agonist sarafotoxin 6b (S6b) inhibited, while the ET_B agonist sarafotoxin 6c (S6c) did not inhibit cytokine-initiated iNOS transcription in MCs. The results demonstrate that ET-1 can strongly inhibit cytokine induction of iNOS and formation of NO in cultured MCs, and that this action is mediated via the ET_A receptor. While the precise mechanism(s) and biological relevance of this ET-1 effect are presently unclear, it is conceivable that down-regulation of iNOS by the vasopressor ET-1 may serve *in vivo* to prevent massive NO build-up and subsequent vasomotor collapse in the glomerular capillary tuft. This could help to maintain glomerular ultrafiltration in states of endotoxin excess as well as during glomerular formation and action of TNF-α and IL-1β causing iNOS induction and subsequent overproduction of NO.

The balance of constricting and relaxing stimuli regulates the tone of the microvasculature and the hydrostatic pressure in the glomerular capillary tuft and, thus, contributes to the control of the glomerular ultrafiltration. Recent studies have shown that nitric oxide (NO) opposes the potent vasoconstrictor action of endothelins (ETs) [1, 2] by generating vasodilatory cyclic guanosine monophosphate (cGMP) [3, 4]. In addition to their constricting effects on vascular smooth muscle cells (VSMCs) of the afferent and efferent arterioles and on glomerular mesangial cells (MCs), ETs are known to stimulate the activity of a constitutively expressed NO synthase (cNOS) of endothelial cells. By increasing the formation of NO, ETs counteract their own vasoconstrictive effect [5, 6]. ET-dependent activation of cNOS and NO release are short-lived and thought to serve the minute-by-minute regulation of the glomerular hydrostatic pressure. In keeping with this concept is the observation that inhibitors of NO formation attenuate a transient depressor effect of ET on arterial blood pressure in rats [7].

Recently, several reports have demonstrated that MCs from different species possess the inducible form of NOS (iNOS) [8–13], which is also found in macrophages and VSMCs [14, 15]. This enzyme generates large amounts of NO for a prolonged time period upon induction by various pro-inflammatory agents and mediators, such as bacterial endotoxin, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The effects of vasoconstrictor substances on the iNOS expression in MCs are presently unknown. Nakayama et al [16] reported that the IL-1β-stimulated expression of iNOS in VSMCs derived from rat thoracic aorta can be inhibited by angiotensin II.

The present study was performed to examine whether ET-1 exerts regulatory effects on the expression of iNOS in cultured rat MCs, as induced by TNF-α plus IL-1β. We found that ET-1 inhibits the formation of iNOS protein and NO by blocking the expression of iNOS mRNA at the level of gene transcription elicited by TNF-α plus IL-1β. This inhibitory effect of ET-1 is mediated via the ET_A receptor, since it can be mimicked by the ET_A receptor agonist sarafotoxin 6b (S6b) and blocked by pretreatment with the ET_A receptor antagonist BQ-123.

Methods

Materials

Human recombinant IL-1β and ET-1 were purchased from Boehringer Mannheim (Mannheim, Germany). Human recombinant TNF-α was a gift from BASF/Knoll (Ludwigshafen, Germany). Sarafotoxins, BQ-123, L-NMMA and chemicals used for the NO_2^- determination were purchased from Sigma (Deisenhofen, Germany). Radioactive products and nylon blotting membranes were obtained from Amersham Buchler (Braunschweig, Germany). Tissue culture plastic was from Falcon (Becton-Dickenson, Heidelberg, Germany), media and sera from Gibco-BRL (Eggenstein, Germany).

Mesangial cell preparation and cell culture

For preparation and culture of glomerular MCs from male Sprague-Dawley rats, standard techniques were used, as described

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Table 1. Synergistic effects of IL-1β and TNF-α on iNOS mRNA induction

	Percent	
IL-1β *U/ml*	Without TNF-α	With TNF-α
0	2.4	2.4
2	3.9	10.2
10	0.6	29.1
25	7.8	58.9
40	5.3	60.6
55	8.3	51.0
70	17.2	72.0
85	9.5	100.0
100	12.5	71.3

MCs were treated with TNF-α (10 ng/ml) and different concentrations IL-1β (0 to 100 U/ml) for 15 hours. Data were calculated from videodensitometric analysis of Northern blots revealing iNOS mRNA expression after probing with ^{32}P-labeled iNOS and reprobing for GAPDH mRNA. Results are given as per cent of maximal ratio of densitometric readings of iNOS mRNA vs. GAPDH mRNA found for IL-1β (85 U/ml) plus TNF-α (10 ng/ml). The data shown are derived from one representative experiment out of three using MC mRNA from two wells per point. Preparation techniques for MCs and mRNA are described in **Methods**.

previously [12, 17, 18]. The outgrowing cells were characterized as MCs by positive immunocytochemical staining for Thy 1.1 (Serotec, Blackthorn, Bicester, UK) smooth muscle cell actin and myosin, showing typical MC morphology [17]. MCs were cultured in Dulbecco's minimal essential medium supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM glutamate, 5 ng/ml insulin, 100 U/ml penicillin and 1 mg/ml streptomycin (all from Sigma). Cells were used for experiments at subconfluence or passaged at confluence with trypsin/EDTA (0.05%/0.02% wt/vol). To obtain quiescent cells, MCs were maintained in medium containing 0.5% FCS for four days before cytokine treatment. MCs were used between passages 8 and 19. In order to find suitable cytokine concentrations for this study, we performed dose-response experiments (Table 1) or chose doses used by us [12, 22] or others [8, 11]. In most experiments, the cytokines IL-1β and TNF-α were used in concentrations of 25 U/ml and 25 ng/ml, respectively.

NO_2^- determination in MC supernatants

MCs were grown to subconfluence in six-well plates and were then conditioned in phenol red-free culture medium for 24 or 48 hours. The NO_2^- content was measured using the Griess colorimetric method [12, 19]. Briefly, 250 μl of supernatant as well as control medium containing known concentrations of $NaNO_2$ were mixed with 50 μl of Griess reagent (25 mM sulphanilamide and 25 mM naphtylethylenediamine) and 25 μl 6 M HCl and incubated for 30 minutes in the dark. Optical density was measured at 550 nm and the NO_2^- content of conditioned medium was calculated from the curve obtained from $NaNO_2$ standards. NO_2^- levels were corrected for the total protein content of MC extracts determined by the Bradford method [20].

Northern hybridization analysis

Total RNA from rat MCs grown to subconfluence in 100 mm culture dishes was obtained, as described [21, 22]. Twenty micrograms total RNA were subjected to electrophoresis in 1% agarose gel containing 1.8% formaldehyde. The separated RNA was transferred to nylon membranes and fixed by baking at 80°C for two hours. Next, the RNA was prehybridized (5 × Denhardt's solution, 5 × SSC, 50 mM Na_3PO_4, 0.1% SDS, 250 μg herring sperm DNA, 50% formamide) for at least two hours at 42°C and then hybridized for 12 to 24 hours using the same conditions. For hybridization, the *Eco*RI/*Pst*I cut insertion of the plasmid piNOS B2 [14], a murine iNOS cDNA clone, was used. This 611 bp fragment, which represents the 5′ region of the iNOS cDNA was ^{32}P-labeled by the use of a random priming kit (Boehringer Mannheim, Germany). The membrane was washed at 42°C (2 × 15 min with 2 × SSC, 0.1% SDS and at least 30 min with 0.2 × SSC, 0.1% SDS) and exposed to X-ray film (Kodak, XAR-5 supplied by Sigma) at −80°C for at least 15 hours. To correct for variations in RNA content, filters were rehybridized with a ^{32}P-labeled rat glycerine-aldehyde-3-phosphate-dehydrogenase (GAPDH) probe, which was obtained using a homology-based reverse transcription-polymerase chain reaction (Gibco, Eggenstein, Germany) of MC mRNA with the following primers added to the reaction mix:

sense: 5′ AATGCATCCTGCACCACCAA 3′
antisense: 5′ GTCATTGAGAGCAATGCCAGC 3′

Determination of iNOS mRNA half life

Quiescent MCs were incubated for eight hours with TNF-α (25 ng/ml) plus IL-1β (25 U/ml) (5 Petri dishes), TNF-α plus IL-1β plus transforming growth factor-β (TGF-β) (5 ng/ml; 5 Petri dishes) or TNF-α plus IL-1β plus ET-1 (10^{-8} M; 5 Petri dishes). Actinomycin D (Act D) (10 μg/ml) was added at different time points (0 to 120 min) before harvesting the cells for total RNA isolation. RNA was electrophoresed and subjected to Northern blot analysis with the labeled cDNA's for iNOS or GAPDH, as described above. Relative iNOS mRNA content was determined calculating the ratio of arbitrary videodensitometrical units obtained for the signals of iNOS versus GAPDH within one blot which was re-exposed several times to ensure linearity of the samples obtained.

Detection of iNOS protein by Western blotting

Preparation of MC lysates and immunoblot analysis was performed using time-matched 100 mm plates of MCs. They were incubated with complete medium containing 0.1% bovine serum albumin without FCS, medium supplemented with TNF-α (25 ng/ml) plus IL-1β (25 U/ml), or medium supplemented with TNF-α plus IL-1β plus ET-1 (10^{-8} M). After 24 hours of incubation, the medium was removed. The plates were washed twice with ice-cold phosphate-buffered saline and 1 ml of boiling lysis solution (1% SDS, 10 mM Tris pH 7.4) was added directly to the cells. The cell lysates were collected in centrifuge tubes and protein contents were determined using the BCA Protein Assay Reagent (Pierce, München, Germany). The samples (50 μg) were diluted in electrophoresis sample buffer (250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol), boiled for five minutes, and resolved by electrophoresis through 0.1% SDS-10% polyacrylamide gels. Proteins were electrophoretically transferred to nitro-cellulose membranes and the transfer efficiency was determined by staining the membranes with Ponceau S. After destaining in distilled water, the membranes were quenched in blocking solution (10% powdered dried low fat milk in washing solution [10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20]) overnight at 4°C. The blocking solution was decanted, membranes were incubated for one hour at room temperature

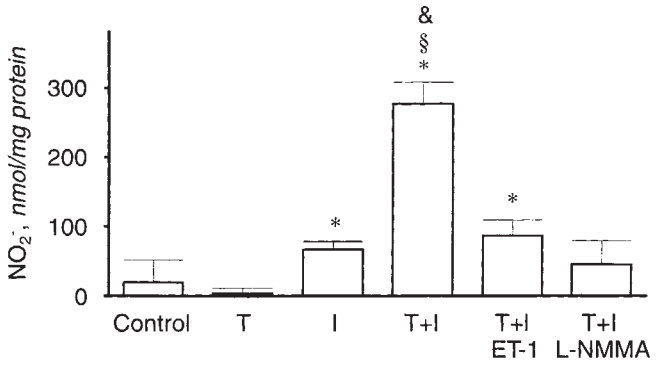

Fig. 1. *NO_2^- production of MCs affected by TNF-α, IL-1β and ET-1.* ET-1 and L-NMMA inhibit the synergistic upregulation of NO_2^- formation by TNF-α plus IL-1β. NO_2^- concentrations were measured in quiescent MCs incubated for 24 hours with the indicated agents (25 ng/ml TNF-α [T], 25 U/ml IL-1β [I], 10^{-8} M ET-1, 1 mM L-NMMA or vehicle as control). NO_2^- concentrations were determined in the supernatant by the Griess reaction. The value was corrected for the total protein content per plate. Data (mean ± SD) represent three (T, T+I+ET-1) or four (C, I, T+I, T+I+L-NMMA) experiments with assays performed in triplicate. $^*P < 0.05$ for I, T+I, T+I+ET-1 versus control; $^§P < 0.05$ for T+I versus I; $^&P < 0.05$ for T+I versus T+I+ET-1.

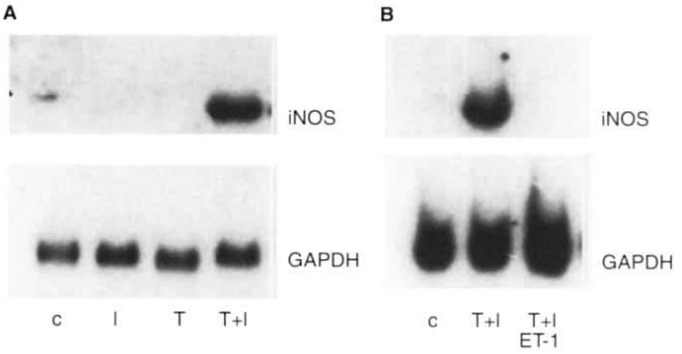

Fig. 2. *Northern blot showing the synergistic effect of TNF-α (T) plus IL-1β (I) on iNOS gene transcription* (**A**) *and inhibition of TNF-α plus IL-1β induced iNOS transcription by ET-1* (**B**). Treatment of MCs for 15 hours with mediators (25 ng/ml TNF-α [T], 25 U/ml IL-1β [I], 10^{-8} M ET-1, or vehicle as control [C]) was followed by cell harvesting for total RNA isolation or protein extraction. RNA was electrophoresed and transferred to nylon membranes. iNOS mRNA was detected by hybridization using ^{32}P-labeled cDNA of the murine iNOS clone piNOS B2. Variation of RNA content of each lane was controlled by rehybridization with a GAPDH cDNA probe.

with an anti-iNOS antibody, raised in rabbits [Affiniti, Nottingham, UK; 1:250 diluted in a second blocking solution (5% powdered dried low fat milk in washing solution)]. Membranes were washed for 30 minutes with several changes of washing solution at room temperature. The bound primary antibody was detected with a secondary antibody as described in the enhanced chemiluminescence protocol (Amersham, Braunschweig, Germany) using a horseradish peroxidase-conjugated anti-rabbit IgG (Serva, Heidelberg, Germany) as secondary antibody (diluted 1:2,000 in the second blocking solution). We also applied a second protocol to detect iNOS proteins using as primary antibody guinea pig IgG directed against murine iNOS protein (provided by S. Bachmann, Heidelberg, Germany) which was diluted 1:250 in the second blocking solution. Goat anti-guinea pig horseradish peroxidase-conjugated IgG (Dianova, Hamburg, Germany) was used, diluted 1:5,000, to detect bound guinea pig IgG using the above protocol.

Statistical analysis

When suitable, means and SDs were determined. Student's *t*-test or analysis of variance (ANOVA), where applicable, were used to test for statistically significant differences (P values < 0.05).

Results

NO_2^- production of cytokine treated MCs

The stable metabolite of NO, NO_2^- was determined in supernatants of cultured MCs treated with IL-1β (25 U/ml), TNF-α (25 ng/ml) or TNF-α plus IL-1β. Supernatants of untreated MCs were used as controls. As shown in Figure 1, incubation of MCs with TNF-α plus IL-1β for 24 hours resulted in a higher NO_2^- content (277 ± 74 nmol/mg protein) compared to IL-1β alone (67 ± 11 nmol/mg protein). TNF-α (25 ng/ml) had no measurable effect (4 ± 8 vs. 8 ± 2 nmol/mg protein of untreated control cells). NO_2^- formation stimulated by IL-1β or by TNF-α plus IL-1β was greatly reduced (46 ± 34 nmol/mg protein) by coincubation with 1 mM of the stable L-arginine analog, L-NMMA, a competitive inhibitor of NO synthesis indicating that NO_2^- formation was dependent on an L-arginine converting enzyme. When MCs were incubated with TNF-α plus IL-1β and additionally with ET-1 (10^{-8} M) for 24 hours, MC formation of NO_2^- was significantly reduced to 87 ± 23 nmol/mg protein.

Effects on iNOS mRNA expression

To test whether the stimulation of NO_2^- production is paralleled by changes of the iNOS mRNA level, Northern blot analysis of iNOS transcription was performed with electrophoretically separated total RNA obtained from MCs incubated with the employed cytokines or vehicle for 15 hours. No iNOS mRNA signal was detected in untreated cells and in MCs incubated with TNF-α (Fig. 2A). IL-1β by itself produced a poorly detectable signal whereas use of both cytokines combined caused strong hybridization showing an iNOS mRNA band at the expected 4 kb size. When increasing concentrations of IL-1β were coincubated with 10 ng/ml TNF-α, maximal iNOS mRNA hybridization signals were detected at a concentration of 85 U/ml of IL-1β (Table 1). In contrast, TNF-α alone had no effect on iNOS induction even when used at concentrations up to 100 ng/ml (data not shown). ET-1 markedly reduced or even abolished the iNOS mRNA signal in MCs treated with TNF-α plus IL-1β (Fig. 2B).

Effects on expression of iNOS protein

Western analysis was performed with two different anti-iNOS antibodies on cell lysates prepared from either untreated MCs, MCs treated with TNF-α (25 ng/ml) plus IL-1β (25 U/ml) or with TNF-α plus IL-1β and ET-1 (10^{-8} M). Combined stimulation with the cytokines showed the expected 130 kD band for the iNOS protein (Fig. 3). No signal was detectable in either the untreated MC lysates or with coincubation of MCs with cytokines and ET-1. This demonstrated that the cytokine-stimulated expression of the iNOS protein paralleled the iNOS mRNA levels and that both were strongly inhibited by ET-1.

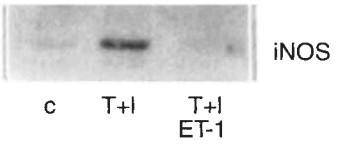

Fig. 3. *Immunoblot analysis for iNOS protein under denaturing conditions.* Lane 1 shows lysates (50 μg protein) of untreated MCs (C), lane 2 shows MCs stimulated with cytokines (25 ng/ml TNF-α, 25 U/ml IL-1β) for 20 hours and lane 3 cytokine-stimulated MCs coincubated with 10^{-8} M ET-1. A 130 kD band was clearly visible only in lane 2, indicating the expression of iNOS protein. The immunoblot shown was obtained by use of an iNOS antibody raised in rabbits (Affiniti, Nottingham, UK). Applying an iNOS antibody raised in guinea-pig (gift from Dr. Bachmann, Heidelberg, Germany) produced comparable bands.

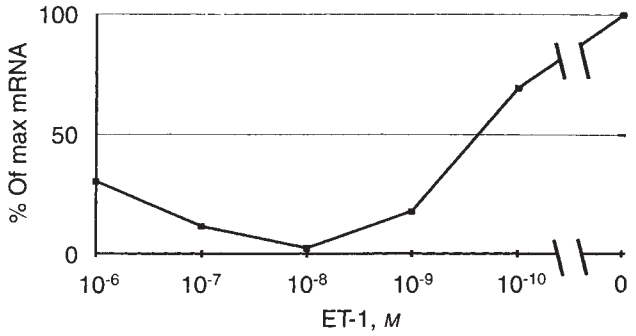

Fig. 4. *Dose response curve of the inhibitory effect of ET-1 on iNOS mRNA transcription.* The signals for mRNA of iNOS and GAPDH from MCs treated for 15 hours with 25 ng/ml TNF-α plus 25 U/ml IL-β and the indicated concentrations of ET-1 were calculated by densitometry. The iNOS/GAPDH ratio of the densitometric readings obtained in the presence of TNF-α plus IL-1β without ET-1 was set to 100%.

Analysis of the inhibition of iNOS mRNA induction

Dose-dependency of the inhibitory effect of ET-1 on iNOS mRNA expression was determined by Northern blot analysis with MCs treated for 15 hours with TNF-α plus IL-1β and coincubated with different concentrations of ET-1. The maximal inhibitory effect was observed at an ET-1 concentration of 10^{-8} M (Fig. 4). Use of shorter periods of coincubation (4 to 10 hr) of ET-1 with TNF-α plus IL-1β showed that a minimum of four hours was required for inhibition of iNOS mRNA induction by ET-1 (data not shown). The action of ET-1 on iNOS induction was further analyzed in the presence and absence of Act D, an inhibitor of RNA synthesis. For comparison with ET-1, TGF-β was also used since Pfeilschifter and Vosbeck [23] have reported that TGF-β inhibits the expression of iNOS when induced by cytokines in rat MCs. MCs were treated with TNF-α plus IL-1β and additionally with either ET-1 (10^{-8} M), TGF-β (5 ng/ml) or with vehicle control during eight consecutive hours. Act D (10 μg/ml) was added at different time points before RNA isolation. The autoradiograms of the Northern blots (Fig. 5B) and the time course of the densitometric readings (Fig. 5A) revealed decreasing iNOS mRNA levels with prolonged Act D treatment. These data indicated that the half-life of MC iNOS mRNA induced by TNF-α plus IL-1β was less than two hours. In the absence of Act D, TGF-β was found to reduce transcription of iNOS to less than 50% of the control level. In addition, TGF-β accelerated the breakdown of transcribed iNOS mRNA during the initial 30 minutes. Since iNOS transcription was almost completely prevented by ET-1 even in the absence of Act D, we were unable to assess an effect of ET-1 on the stability of iNOS mRNA.

Analysis of ET receptors

At least two ET receptors have been reported to mediate ET-1 induced effects in target cells [24, 25]. We used the ET_A receptor-selective agonist S6b and the ET_B receptor-selective agonist S6c [26, 27] as well as the ET_A receptor antagonist BQ-123 [28] to examine the receptor specificity of the inhibitory effect of ET-1 on iNOS mRNA transcription in MCs. As shown in Figure 6A, S6b and ET-1 (in concentrations between 10^{-9} and 10^{-7} M) completely abolished the iNOS mRNA signal induced by TNF-α plus IL-1β. By Northern blotting, S6c showed neither significant inhibition nor stimulation of iNOS transcription (Fig. 6). This was assessed by densitometric evaluation of iNOS versus GAPDH mRNA in five separate experiments. For the representative blot shown in Figure 6A, the densitometric ratios iNOS versus GAPDH were for S6c (10^{-7} M) 0.78, for S6c (10^{-8} M) 0.52 and for the control (T+I) 0.78. The effect of S6b (no iNOS signal detectable) was partly reversible by preincubation for one hour with the ET_A receptor antagonist BQ-123 (10^{-7} M; iNOS/GAPDH ratio = 0.69). Overexposure of the iNOS mRNA-labeled filter revealed a partial reversion of the inhibitory ET-1 effect by BQ-123 more clearly (Fig. 6B).

Discussion

Contractile MCs play an important role in the regulation of glomerular hemodynamics and ultrafiltration [29]. Under physiological conditions, glomerular blood flow, hydrostatic pressure, and ultrafiltration are tightly regulated by the balance and interplay of vasoconstrictors (such as angiotensin II and ETs) and vasodilators (such as prostaglandins and NO). It is well known that MCs produce and are responsive to vasoactive substances as well as to inflammatory cytokines. During glomerular inflammation, multiple cytokines have been described to affect the MC phenotype. IL-1β and TNF-α are known to induce many biological responses in MCs, such as they stimulate MC proliferation and cause formation and release of autocrine mediator substances including NO [8, 12, 29, 30]. NO has been shown to affect the phenotype of MCs in culture and *in vivo* which, in turn, may influence the function of the glomerular capillary ultrafiltration apparatus.

Besides beneficial effects, such as the prevention of intraglomerular thrombosis [31], induction of iNOS by endotoxin or cytokines followed by prolonged NO production could also be detrimental for glomerular function and structure. For example, massive NO release following induction of iNOS could lead to a marked relaxation of glomerular MCs as well as VSMCs of the afferent and efferent arteriole causing reduction of glomerular hydrostatic pressure and ultrafiltration. ET-1 is a strong pressor substance secreted by the vascular endothelium. ET-1 opposes the vasodilatory effect of NO by direct vasoconstriction. Conceivably, ET-1 may also prevent inappropriate vasodilatation due to cytokine-stimulated excess NO formation within the glomerulus by downregulating iNOS expression in MCs. To investigate this question we studied the effects of ET-1 on the induction of iNOS caused by TNF-α and IL-1β in cultured MCs.

Synergistic effects of TNF-α and IL-1β on iNOS expression and formation of NO in MCs have been reported previously [8, 32]. In

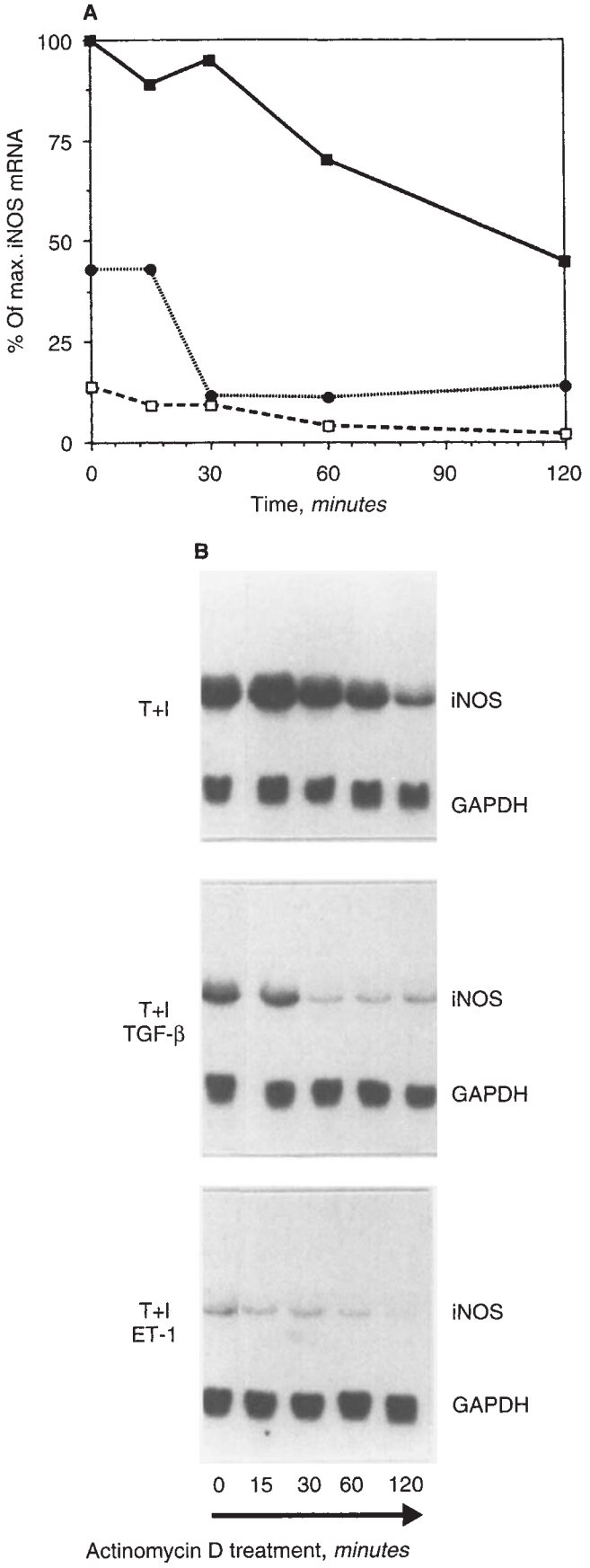

Fig. 5. *Comparison of the inhibitory effects of ET-1 and TGF-β on iNOS mRNA transcription induced by TNF-α plus IL-1β.* Quiescent MCs were treated with TNF-α (25 ng/ml) plus IL-1β (25 U/ml) (■, T + I) and additionally with either ET-1 (10^{-8} M; T + i + ET-1, □) or TGF-β (5 ng/ml; T + i + TGF-β, ●) for eight hours. Act D (10 μg/ml) was added at the indicated time periods before cell harvesting, RNA isolation and Northern blot analysis of total RNA. The intensities of the resulting bands were quantified by videodensitometry and ratios of iNOS/GAPDH bands were calculated setting maximal iNOS transcription induced by TNF-α plus IL-1β without inhibitors to 100% (**A**). The autoradiograms of the blotted RNA's, hybridized with piNOS B2 or GAPDH are displayed in (**B**). The data shown represent one of two similar experiments.

the MC culture system used in the present study, induction of iNOS was not detectable for TNF-α and was weak for IL-1β, as long as these cytokines were administered separately. However, coincubation of MCs with both cytokines led to strong expression of iNOS mRNA and protein. Synergistic upregulation of iNOS in various types of cultured rat or human cells by two or more cytokines (with or without endotoxin) has been observed by many investigators. These effects are commonly explained by the assumption that more than one pathway is necessary to yield maximal iNOS induction by cytokines [11]. Using coincubation of MCs with TNF-α plus IL-1β for up-regulation of iNOS mRNA, we investigated the effects of ET-1 on iNOS expression in MCs.

We found that ET-1 inhibited cytokine-induced iNOS expression on the mRNA level in MCs in a dose-dependent fashion. The inhibitory effect was observed when ET-1 was given simultaneously or at least two hours before and up to five hours after cytokine treatment (data not shown). These findings strongly argue for a specific effect of ET-1 on iNOS expression and against ET-1 exerting inhibitory effects through changes in cytokine receptor expression. The production of NO by MCs was also greatly reduced by ET-1. Indeed, we observed that ET-1 inhibits NO_2^- accumulation in the MC culture supernatant to a similar extent as caused by the inhibitor of NOS activity, L-NMMA (Fig. 1).

To gain further insight into the mechanism of iNOS inhibition elicited by ET-1, we compared its action to that of TGF-β, for which posttranscriptional inhibitory effects on iNOS expression have recently been described [23, 33]. The almost complete prevention of iNOS mRNA induction in the presence of ET-1 indicates that ET-1 suppressed transcription of iNOS mRNA or led to unstable iNOS mRNA with rapid degradation. An additional post-transcriptional effect, causing further down-regulation of the iNOS mRNA seems unlikely since ET-1 was unable to induce degradation of transcribed iNOS mRNA. In preliminary studies, we found that ET-1 when given 15 to 22 hours after the addition of TNF-α plus IL-1β did not diminish iNOS mRNA content over a period of one to eight hours (data not shown). Inhibition of iNOS mRNA expression by TGF-β in endotoxin-stimulated murine macrophages has been shown to occur at transcriptional as well as post-transcriptional levels [33]. In our experiments, TGF-β caused partial inhibition of TNF-α plus IL-1β-induced transcription of iNOS mRNA. In addition, in the presence of TGF-β, we observed accelerated degradation of the transcribed iNOS mRNA. Since TGF-β up-regulates prepro-ET-1 gene expression, the possibility exists that TGF-β down-regulates iNOS expression in part through ET-1 REF [34]. In this study, we did not measured ET-1 produced endogenously by MCs. Under

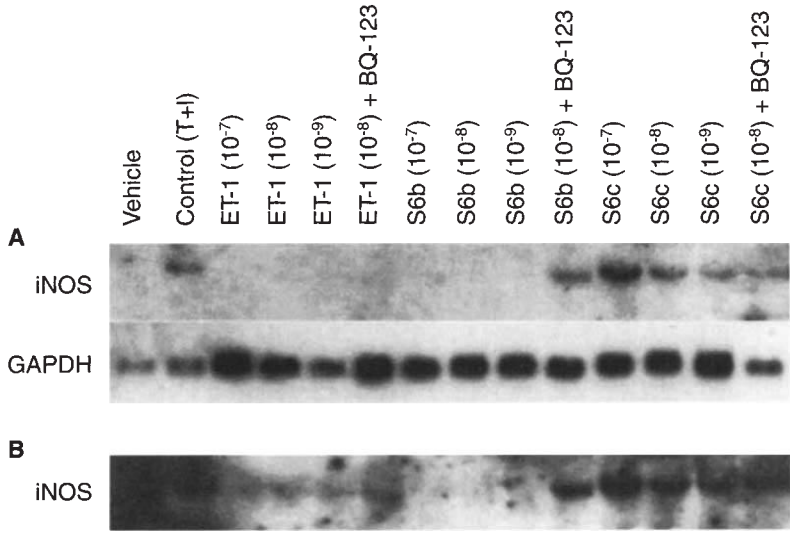

Fig. 6. *Influence of the ET receptor agonists S6b and S6c and the ET_A receptor antagonist BQ-123 on iNOS transcription induced by TNF-α plus IL-1β (T + I).* Quiescent MCs were treated with TNF-α (25 ng/ml) plus IL-1β (25 U/ml) and additionally with the indicated agents for 15 hours. BQ-123 was added one hour before the other agents. Total mRNA was subjected to Northern blotting. Equivalent loading of the gel was verified by reprobing blots with GAPDH cDNA. Normal exposure of membrane bound radioactivity for one day is shown in (**A**). Prolonged exposure for three days (carried out to better visualize the reversibility of ET-inhibition by BQ-123) is shown in (**B**).

the experimental conditions used, we found no evidence that ET-1 exerts a post-transcriptional effect to regulate NO production beyond the iNOS mRNA level. The inhibition of iNOS transcription by ET-1 is also supported by the observation that reduction of iNOS protein expression and iNOS activity (as assessed by NO_2^- accumulation) paralleled iNOS mRNA levels (Figs. 1 and 2).

Recently, Owada et al [35] have shown that activation of the ET_B receptor by ET-3 or by the selective ET_B agonist IRL 1620 increased cGMP formation in cultured rat MCs in the presence of 20% FCS. This effect reached a maximum within five minutes after addition of the agonists and was inhibitable by L-NMMA. The authors concluded that calcium-dependent NO release was responsible for cGMP formation and that a ET-3-activated, calmodulin-dependent cNOS isoform is present in MCs. The MCs used in our study also reacted to the ET_B selective agonist S6c with increased NO_2^- production (inhibitable by L-NMMA) as long as they were maintained in medium containing 10% FCS (data not shown). This confirms the results of Owada et al [35] indicating the presence of ET_B receptors and a constitutive NOS in growing MCs. However, we were unable to detect NO_2^- formation in quiescent MCs which were maintained in 0.5% FCS for four days before incubation with S6c (data not shown). It is possible that additional growth factors which are present in 10% FCS or produced by growing MCs are necessary to permit NO generation elicited via the ET_B receptor. The ET_A selective agonist S6b showed no effect on NO_2^- production independent from the presence of FCS (data not shown). However, S6b inhibited iNOS transcription induced by TNF-α plus IL-1β in a manner similar to ET-1, indicating that the ET_A receptor is responsible for this effect. The available data support the concept that ETs act on MCs via two different receptors. First, by activating a constitutive, calcium and calmodulin-dependent NOS via the ET_B receptor [35]. This activation and subsequent NO formation is short-lived and subject to tight control. Second, by inhibiting a cytokine-induced calcium and calmodulin-independent NOS via the ET_A receptor preventing the production of large amounts of NO over longer time periods.

In summary, our findings in MCs demonstrate that the vasoconstrictor ET-1 can block the induction of iNOS by cytokines and prevent the excess formation of vasorelaxant NO. The inhibition is mediated via the ET_A receptor. The precise mechanisms of action and the biological relevance of the inhibitory effect of ET-1 on iNOS expression and NO production are presently unclear. With regard to maintenance of the glomerular microcirculation, it is conceivable that this action of ET-1 may serve to protect glomerular ultrafiltration by inhibiting prolonged production of large amounts of vasodilatory NO by MCs. This may be of relevance in states of endotoxin excess with systemic or local overproduction of TNF-α and IL-1β, as seen in sepsis and acute glomerular inflammation.

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